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Novel Method to Assess Arterial Insufficiency in Rodent Hindlimb

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Abstract

Background—Lack of techniques to assess maximal blood flow capacity thwarts the use of rodent models of arterial insufficiency to evaluate therapies for intermittent claudication. We evaluated femoral vein outflow (VO) in combination with stimulated muscle contraction as a potential method to assess functional hindlimb arterial reserve and therapeutic efficacy in a rodent model of subcritical limb ischemia.

Materials and methods—VO was measured with perivascular flow probes at rest and during stimulated calf muscle contraction in young healthy rats (Wistar Kyoto, WKY; lean Zucker, LZR) and rats with cardiovascular risk factors (Spontaneously Hypertensive, SHR; Obese Zucker, OZR) with acute and/or chronic femoral arterial occlusion. Therapeutic efficacy was assessed by administration of Ramipril or Losartan to SHR after femoral artery excision.

Results—VO measurement in WKY demonstrated the utility of this method to assess hindlimb perfusion at rest and during calf muscle contraction. While application to diseased models (OZR, SHR) demonstrated normal resting perfusion compared to contralateral limbs, a significant reduction in reserve capacity was uncovered with muscle stimulation. Administration of Ramipril and Losartan demonstrated significant improvement in functional arterial reserve.

Conclusion—The results demonstrate that this novel method to assess distal limb perfusion in small rodents with subcritical limb ischemia is sufficient to unmask perfusion deficits not apparent at rest, detect impaired compensation in diseased animal models with risk factors, and assess

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therapeutic efficacy. The approach provides a significant advance in methods to investigate potential mechanisms and novel therapies for subcritical limb ischemia in pre-clinical rodent models.

Keywords

arterial insufficiency; arterial reserve capacity; arterial occlusion; pre-clinical models; venous flow

1. Introduction

Peripheral arterial disease (PAD) of the lower extremity is a significant and serious health problem associated with increased morbidity, mortality and decreased quality of life(1, 2). It afflicts ~8 million Americans(3), including ~20% of the population over age 55(4) and ~60% of those ≥ 85 years(5, 6). Its prevalence is increasing significantly(7, 8) as the population lives longer with chronic disease and risk factors such as diabetes. Approximately 10–20% of the population with PAD eventually experience pain during walking (intermittent claudication, IC) due to arterial insufficiency or inadequate perfusion during increased metabolic demand(5, 9). Within 10 years of diagnosis with IC, 20 – 30% progress to critical limb ischemia (CLI) characterized by rest pain or tissue loss (ulceration or gangrene) generally in the foot. This number increases to 45 – 60% if the patients are diabetic(10).

Based upon these statistics, there is widespread agreement that PAD is a malady in need of new medical treatments. Indeed, the development of therapies to improve function and quality of life in all stages of PAD has been identified as a great and unmet medical need(11, 12). While multiple clinical trials with CLI patients have been conducted in recent years, few clinical studies have focused on the much more prevalent condition of IC. Moreover, there is a significant lack of medications effective in relieving symptoms and improving function in claudicants(13). Recent clinical reports using Ramipril (12, 14) have demonstrated the potential of pharmacological therapy to significantly improve muscle function and perfusion in claudicants. While the results are impressive and promising, significant questions have been raised regarding mechanisms and drug specificity(13). Consequently, additional studies are warranted. Furthermore, appropriate pre-clinical studies could provide important direction and insight regarding mechanisms and novel treatments for claudicants. However, the evaluation of potential therapeutics to improve subcritical limb ischemia requires assessment of maximal blood flow capacity or flow reserve, and the lack of such techniques in small animal models is a major limitation for their use in preclinical studies(15–17).

The purpose of this study was to evaluate femoral vein outflow (VO) in combination with stimulated muscle contraction as a potential method to assess functional hindlimb arterial reserve and therapeutic efficacy in a small rodent model of subcritical limb ischemia. Distal femoral vein outflow was measured with ultrasound transit time perivascular flow probes at rest and during electrically stimulated skeletal muscle contraction to assess functional reserve capacity. We first established the potential utility of this technique in young, healthy rats. Then, rats with vascular risk factors and known impairments to vascular compensation were used to assess the ability of this technique to detect perfusion deficits that were not

apparent at rest. Finally, the ability of this method to assess potential therapeutic efficacy was tested in rats with risk factors that also received pharmacological therapy.

1. Methods

1.1 Animals

These animal studies were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee. The animal care was in compliance with the *Guide for the Care and Use of Laboratory Animals.* Only male animals were used to minimize variability in these experiments to assess the feasibility and utility of the venous outflow technique.

The first series of experiments were performed in young Wistar Kyoto rats (WKY, 8–10 wks old) obtained from Harlan Laboratories, Inc. (Indianapolis, IN) to assess the feasibility of measuring venous outflow, in the right hindlimb 2 wks after femoral artery ligation (Ligated) and in the left limb before (Non-Occluded) and after acute clamping (Acute) of the distal femoral artery (Figure 1), both at rest and during electrically induced calf muscle contraction.

After establishing feasibility, the next experiments were performed to evaluate the utility of this technique to assess differences between the diseased models of obese Zucker rats (OZR, Charles River Laboratories, Wilmington, MA) and retired breeder Spontaneously Hypertensive rats (SHR, Harlan Laboratories Inc.) compared to lean, normotensive control rats (lean Zucker rats, LZR). The OZR and SHR were selected because they are the most common rat strains utilized in studies of hindlimb ischemia(18–31) and have disorders typically associated with vascular disease in humans. We used femoral artery excision in these animals as most previous studies in these strains have utilized this method to induce arterial insufficiency. In these animals, venous outflow was measured in both left and right hindlimbs at rest and during electrically induced calf muscle contraction, 2 wks after right femoral artery excision (Excised). In the left limb the measurements were made before (Non-Occluded) and after acute (Acute) clamping of the distal femoral artery (Figure 1).

The final set of rat experiments was conducted to evaluate the ability of the venous outflow technique to assess the efficacy of potential therapies. SHR were treated with renin-angiotensin system suppressing agents because this class of drug has been shown to provide remarkable benefit to human claudicants with hypertension(12, 14) and has demonstrated similar effects in prior rodent studies(28, 32). Treatment was initiated 2 weeks after femoral artery excision and venous outflow was measured in the right hindlimb of SHR two weeks later (4 weeks post-excision).

Lastly, acute studies were performed in mice to evaluate the potential application of venous outflow studies during skeletal muscle stimulation in this species. Adult C57BL/6 mice ≥12 weeks of age were obtained from an established colony.

2.2 Model creation of arterial insufficiency

2.2.1 Femoral artery ligation in rats—In the initial studies to develop the protocol for the venous outflow method and test the feasibility of unmasking a perfusion deficit with electrically stimulated skeletal muscle contraction, a single ligation of the femoral artery was performed in WKY rats. Following induction of anesthesia with isoflurane, a transverse incision (~2 cm) was made below the right inguinal ligament. Under microscopic observation and using fine forceps, a short segment of the right femoral artery distal to the superficial circumflex iliac artery (Figure 1) was exposed, carefully isolated from the nerve and vein by placing tension only on the artery or surrounding fascia, and then ligated. Tissues were kept moist with sterile saline during the entire procedure. The skin incisions were then closed and the animals recovered.

2.2.2 Femoral artery excision in rats—Femoral artery excision results in a more robust model of arterial insufficiency and was used to allow comparison with previous studies of hindlimb vascular compensation in LZR, OZR, and SHR rodents(19, 21, 30). As described by Emanueli et al. (21), under anesthesia an incision was made from the inguinal ligament to the knee. With microscopic observation and using fine forceps to contact only the artery or surrounding fascia, the right femoral artery was exposed and isolated from the circumflex iliac artery to the saphenous and popliteal bifurcation. Both ends of this segment were ligated as well as all branches. The femoral artery was then excised, the incision closed, and the rats recovered. Tissues were kept moist with sterile saline during the entire procedure.

2.3 Pharmacological therapy

Three groups of SHR rodents with femoral artery excision, as described above, received untreated drinking water (controls) or water treated with Ramipril (Sigma Chemical Co.) or Losartan (kindly provided by Merck) at 0.2 μM and 0.6 mM, respectively, beginning 2 weeks post-excision. Following treatment for two weeks, hindlimb flow studies were conducted to assess the effect of therapy.

2.4 Perfusion assessment

Laser Doppler perfusion imaging (LDPI, Model LD12-IR, Moor Instruments LTD, Millwey, UK) was performed of the plantar surface of the hind paws under gaseous anesthesia with isoflurane to monitor recovery following ligation, as previously reported(33).

Femoral venous outflow measurement during skeletal muscle contraction was performed as follows. Rats were anesthetized with intraperitoneal Nembutal (50 mg/kg) and supplemented as needed. A tail vein catheter was placed for hydration and administration of supplemental anesthetic infusion. In addition, a tracheostomy was established and mechanical ventilation initiated and maintained. A right common carotid artery line was placed for continuous monitoring of mean arterial pressure (MAP). The femoral vein was carefully dissected under microscopic observation using fine tipped forceps to touch only surrounding tissues. Zero or baseline flow values were established with the flow probe immediately adjacent to the vein. Without contacting the vein with the forcep tips, sutures were placed loosely around the vein and used to assist in positioning the vein within the perivascular ultrasound transit time flow probe (Transonic Flowprobe, Transonic Systems Inc., Ithaca, New York, USA). Femoral

vein flow measurements were recorded during both resting conditions and contraction-induced hyperemia brought on by electrical stimulation. Measurements in the left limb were made both before and after acute clamping of the left femoral artery. For electrical stimulation, surgical suture needles were placed anterior to the Achilles tendons and within the medial thigh musculature on each limb and connected to an electrical stimulator (Grass SD9 Stimulator, Grass Technologies, West Warwick, RI, USA). A setting of 7.0 volts was used for these studies, with a duration of 0.6 milliseconds. Femoral venous outflow was continuously recorded at variable contraction frequencies for 3 minutes each.

2.5 Acute feasibility studies in mice

Mice were anesthetized to effect via isoflurane mixed with oxygen. Following a 1.5 cm incision in the overlying skin from the level of the epigastric artery to the inguinal ligament, the nerve, artery, and vein were gently separated with fine tip forceps while tension was placed on the surrounding fascia. Extreme care was taken to minimize contact of the vein and nerve. Loops of 6–0 silk suture were then placed around the artery and vein to facilitate placement of the vessel within the flowprobe (Transonic 0.5PSB nanoprobe) without damage. The flowprobe was attached to a micromanipulator to allow fine adjustments under microscopic observation to ensure the vein was completely unobstructed and that the artery was not compressed. After obtaining a baseline flow, 0.125mm silver wires (WPI, Inc.) were inserted into the gastrocnemius muscle (where it becomes visible below adductors) and at the same level on the opposite side of the saphenous vessels. After obtaining venous flow under baseline and stimulated levels, the femoral artery was occluded just distal to the origin of the superficial epigastric artery and measurement of baseline and stimulated flow was repeated.

2.6 Statistical analyses

All data are expressed as means (±SEM). The statistical methods used throughout this study involved 2-way repeated measures (within subject) ANOVA unless otherwise specified in text or legends. When the ANOVA indicated statistical significance between group differences, the Holm-Sidak method was used for pairwise multiple comparison procedures. Values of $P < 0.05$ were considered significant.

3. Results

Average body mass and mean arterial pressures for the various experimental groups are reported in Table I. Body mass and arterial pressures were higher in OZR than LZR and arterial pressure was reduced by Ramipril and Losartan treatment in the SHR.

3.1 Assessment of the technique in young healthy animals

3.1.1 Feasibility of measuring venous outflow from the lower limb—Figure 2A depicts results obtained in WKY with the transit time ultrasonic flow probe positioned first on the distal femoral vein and then on the artery of the same rat limb. The tracing shows virtually identical blood flows when measured in the artery and vein both at rest and during functional hyperemia induced by stimulated muscle contraction. Similar results were

observed in two animals and demonstrated the feasibility of assessing perfusion of the lower limb by measurement of venous outflow both at rest and during hyperemia.

3.1.2 Effect of femoral artery ligation on venous outflow—The tracings in Figure 2B illustrate typical changes in venous outflow observed at rest and during skeletal muscle contraction at 6 Hz in the left hindlimb before (Non-Occluded) and after acute clamping (Acute) of the femoral artery and in the right limb 14 d post-ligation of the femoral artery. Average values for venous outflow at rest (0 Hz) are shown in the first set of bars in Figure 2C. With acute clamping of the femoral artery, femoral vein blood flow at rest was reduced to 54±4.8% of the non-occluded value (P=0.004). Resting flow in the ligated limb was 72±9.2% of the same animal control limb without femoral artery occlusion and tended to be different than the flow measured without arterial occlusion (P=0.065), but was not statistically different than the flow observed after acute clamping (P=0.21).

3.1.3 Effect of femoral artery ligation on contraction-induced hyperemia—The second through fourth sets of bars in Figure 2C show venous flow measurements as the skeletal muscle is contracted at 2, 6, and 12 Hz. Blood flow increased from rest at all stimulus levels in limbs without femoral artery occlusion (Non-Occluded). With acute clamping of the left femoral artery (Acute) and previous ligation of the right (Ligated), flow was significantly increased at 6 and 12 Hz; but the increase was substantially less than observed in the non-occluded condition. For example, paired comparisons of limbs within animals indicated that whereas blood flow in the control limb increased by 505±36.6% at maximal stimulation (12 Hz), it only increased 105±35.7% and 119±35.6% in acutely clamped and ligated limbs, respectively. The difference in femoral venous flow between the Acute and Ligated limbs is statistically significant during skeletal muscle contraction at 6 and 12 Hz, indicating vascular compensation had occurred. Taken together, the data indicate that functional hyperemia in response to skeletal muscle contraction is severely limited with acute occlusion and at two weeks post-ligation.

3.1.4 Compensation to femoral artery ligation assessed by LDPI—Chronic compensation to femoral artery ligation in the young WKY as assessed by LDPI is shown in Figure 2D. LDPI demonstrated an initial drop in perfusion of the ligated limb to an average of 39±2.2% of the control limb on post-op day 1. Over the course of the study, perfusion in the ligated limb improved showing statistically significant increases from days 1 to 3 and 3 to 7. The resting perfusion relative to the control limb reached a plateau at day 7 (65±1.7%) as there was no further increase at day 14 (67±3.9%, P=0.48). These results are similar to those obtained at rest with the venous outflow method, but provide no insight into the functional or maximal reserve capacity of limbs with arterial occlusion relative to control limbs.

3.2 Assessment of the technique in models of arterial disease

3.2.1 Compensation to femoral artery excision assessed by LDPI—The results of LDPI assessment of perfusion from 1 to 14 days post-excision are presented in Figure 3A. Two-way repeated measures ANOVA indicated statistically significant differences in all days post-op, but no differences between groups and no interaction (day x treatment group)

effect. On the first day post-excision, LDPI results show a ratio of experimental to control limb perfusion of 0.395 ±0.040, 0.345 ± 0.029 and 0.648 ± 0.060 for LZR, OZR, and SHR, respectively. By post-op day 14, perfusion had increased to a similar level in all groups.

3.2.2 Effects of femoral artery excision on venous outflow at rest and during contraction induced hyperemia—Average femoral vein flow is shown under resting conditions (0 Hz) and during muscle stimulation at 2 and 12 Hz for LZR, OZR and SHR in Figure 3 B, C, and D, respectively. In control limbs without femoral artery occlusion (Non-Occluded), venous flow increased significantly during muscle contraction in all rats (LZR, OZR and SHR) and flow at 12 Hz was greater than 2 Hz. With acute femoral artery occlusion, venous flow at rest (0 Hz) was significantly depressed in all groups. Resting flow in the limbs with femoral artery excision was similar to flow in the non-occluded limb of all groups. Venous flow at rest was higher in the limbs with femoral artery excision than acute clamping in LZR and SHR, but not OZR (P=0.109). The skeletal muscle contraction component of the study revealed profound perfusion impairment in the limbs with both acute clamping and excision for all groups--; no increase in venous flow was observed after acute clamping during 2 or 12 Hz stimulation. With maximal stimulation (12 Hz), flow in limbs with femoral artery excision increased by 112 ± 22.6% in LZR, 22 ± 10.4% in OZR, and 104 ± 20.6% in SHR compared to 230 ± 42.7%, 161 ± 16.8%, and 255 ± 44.4% respectively for limbs without occlusion. In limbs with excision, the relative increases from rest were statistically greater in LZR than OZR. It is important to note that the very significant perfusion deficit in the chronic limbs was only apparent under hyperemic conditions. It should also be noted that while comparison of the limbs 2 weeks after femoral excision to limbs with acute clamping of the femoral artery indicate compensation has occurred, the degree of compensation is likely underestimated as the initial degree of arterial insufficiency would be less with acute clamping than femoral excision.

3.3 Assessment of therapeutic efficacy: Effects of Ramipril and Losartan Post-treatment on contraction-induced hyperemia in SHR limbs with femoral artery excision

To assess the effect of pharmacological therapies for enhancement of compensation to arterial occlusion, venous outflow with electrical stimulation studies were performed in the SHR hindlimbs using an angiotensin converting enzyme inhibitor (Ramipril) and an angiotensin type I receptor blocker (Losartan). Drug therapy was initiated 2 weeks after femoral artery excision and continued for 2 weeks. Results are presented in Figure 4. Resting venous flows were similar between all groups and were increased significantly in all groups from 0 to 2 Hz and 2 to12 Hz. Relative to the untreated group, flow during muscle contraction was significantly greater in the Ramipril treated group at 2 and 12 Hz and in the group receiving Losartan at 12 Hz. Comparison of the Ramipril and Losartan groups indicated a statistically significant difference at 12 (p=0.03) but not 2 Hz (p=0.10).

4. Discussion

In this work we have addressed the need for more robust methods for the assessment of vascular compensation in the hindlimb of small rodents which have near normal perfusion at rest, but demonstrate a suppressed ability to increase perfusion during muscle activity; a

condition referred to as subcritical limb ischemia. We have presented a novel combination of venous outflow and contraction-induced skeletal muscle hyperemia and demonstrated that this method is capable of unmasking substantial perfusion deficits that are not apparent at rest. Our results show the utility of this technique in identifying differences between healthy rats versus rats with vascular risk factors and in assessment of therapeutic efficacy. Specifics regarding the development of the technique and its advantages and limitations over existing methods as well as the potential significance of the results obtained in diseased rats are considered below.

4.1 Development and evolution of the model

Venous outflow has been utilized in previous studies with larger animals to assess vascular compensation to arterial occlusion. After external iliac artery occlusion in dogs, Coffman (34) assessed collateral blood flow and reactivity by measurement of external iliac venous outflow via a continuously recording rotameter. After confirming that arterial inflow equaled venous outflow in his model, he obtained venous outflow measurements at rest, during contraction of the thigh muscles by electrical field stimulation and with infusion of vasoactive substances. Sanne and Sivertsson in 1968 (35) utilized venous outflow assessed by an optical-drop method at rest and during maximal peripheral dilation to quantify vascular compensation to femoral artery ligation in the cat. Although not previously utilized for this purpose to our knowledge, the development and miniaturization of transit time ultrasound flow probes (36) has made it possible to perform similar venous outflow studies in the rat and mouse hindlimb(37–40). These probes have been shown to provide accurate measurements of volume flow on arteries and veins as determined by roller pumps and volume collections as well as microsphere and clearance techniques(36, 41–44). We have utilized this approach with electrical field stimulation, based on parameters previously shown to be specific to the skeletal muscle cells and motor neurons(45–47) to assess the capacity for functional hyperemia (i.e. increased perfusion with elevated metabolic demand). Our results (Figure 2) demonstrate that distal venous outflow and arterial inflow are equivalent and that this combination can provide a continuous, real-time measurement of flow throughout the period of hyperemia to allow determination of average, total, and peak blood flow for various stimulus levels. Both the extent of impairment and degree of compensation can be assessed by comparing the venous outflow of the control limb before and after acute clamping of the femoral artery to the chronic experimental limb within the same animal (Figure 2C). The results we obtained in young healthy animals are similar to what others have reported utilizing different methodologies. In the control limbs we observed venous flow to increase approximately 5-fold, which is similar to the magnitude of the increase measured by microsphere technique in the rat gastrocnemius muscle with peroneal nerve stimulation(16) and rabbit extensor digitorum longus muscle with electrical field stimulation(48). While direct comparisons of the experimental limb in the current and previous studies cannot be made due to differences in ligation models, anesthesia, as well as species and strains, all studies demonstrate that muscle contraction unmasks a perfusion deficit that is not apparent at rest.

4.2 Application to Models with Arterial Disease

Investigators have realized the potential importance of utilizing animal models of vascular disease in studies of arterial insufficiency, with the hope of more closely approximating the human situation(49). We thus sought to apply our measurement techniques to two rodent models of disease; the OZR and the SHR. Both of these strains have been documented to have a sustained impairment in hindlimb perfusion relative to non-diseased control animals after femoral artery excision (21, 22, 30, 31). Unlike these previous studies, we did not observe a perfusion deficit under resting conditions after recovery in the experimental limb as assessed by either LDPI or venous flow measurements (Figure 3). This might be explained by subtle differences in the excision model or surgical technique and associated injury and/or the anesthetics used. Alternatively, LDPI in the hindlimb is characterized by significant variation between animals and even control groups within a study(50, 51). We did observe a significant flow impairment with electrical hindlimb stimulation in the experimental limb of WKY, LZR, OZR, and SHR. Furthermore, our data reveal that while LZR, OZR, and SHR all exhibited the ability to significantly increase flow during simulated exercise in the experimental limb with chronic femoral artery excision, the increase was significantly less in OZR and SHR than LZR. The results confirm the utility of the venous outflow method with electrical stimulation to unmask perfusion deficits and also suggest the method could be useful to investigate the mechanisms for impaired compensation in models of subcritical limb ischemia with vascular risk factors.

4.3 Evaluation of Therapeutic Assessment

Having established the existence of subcritical limb ischemia in the SHR and OZR, we next sought to determine if the venous outflow method could be utilized to assess potential therapeutic efficacy in a model of subcritical ischemia. We evaluated the effect of Ramipril in the SHR because angiotensin converting enzyme inhibitors have been shown to improve perfusion in the SHR hindlimb(21), promote collateral growth in the SHR mesentery(28), and more importantly improve pain free and maximal walking times in human claudicants(12). We also chose to evaluate the angiotensin type 1 receptor antagonist Losartan, as it is a commonly prescribed drug which might have positive or negative effects on vascular compensation to arterial occlusion(18, 52, 53). Our results (Figure 4) demonstrate that neither Losartan nor Ramipril treatment significantly altered resting perfusion, but both substantially enhanced blood flow during muscle contraction. It is also important to note that the therapies were initiated 2 weeks after arterial occlusion; a post-occlusion time point at which previous rat studies have shown hindlimb perfusion to have reach a plateau (21, 24) and at which recovery to control limb resting perfusion was observed in the current study(Figure 3A). It is also significant that the work by Emanueli et al. in SHR with femoral artery excision(21) found no increase in capillary or arteriolar density with Ramipril treatment that produced improvement in resting perfusion assessed by LDPI. These data suggest that the primary compensation might occur by collateral compensation. Our work also suggests that Ramipril is more efficacious than Losartan, a result that could be explained by the effect of ACEI on nitric oxide bioavailability(54). The results clearly indicate that this approach can establish therapeutic efficacy even when not commenced until after adaptations have restored resting perfusion to control levels. This addresses a major concern regarding whether efficacy of an intervention can be determined

if it is not administered before significant compensation has occurred(15) and also indicates an urgent need for additional studies to identify the specific mechanisms through which the benefits of Ramipril therapy is mediated.

4.4 Advantages and limitations

In their review of models and techniques for preclinical studies of peripheral arterial disease, Waters et al. (15) provided both general and specific recommendations which we considered in the development of our method. A major recommendation related to the need for measurement of collateral-dependent blood flow was direct measurement of blood flow to the distal hindlimb during local dilation so that the dominant resistance was that of the collateral circuit. However, there is currently no ideal method to assess collateral dependent perfusion in small rodents.

Lofti et al. (55) have reviewed the perfusion assessment methods that have been used in studies of hindlimb ischemia including LDPI, arterial flow probes, and microspheres. LDPI is the most commonly used technique for perfusion and offers major advantages in that it is non-invasive, can be used for longitudinal studies, and is relatively easy to learn(55). However, it is primarily used for assessment of resting flows and is considered to be inadequate to assess vascular reserve or collateral function/adaptation(15, 56). While Corcoran et al. (17) have attempted to assess reserve flow with LDPI by arterial occlusion to induce reactive hyperemia, an increase of only ~60% was observed in the control limb, which is far less than the normal flow reserve for skeletal muscle (16, 57–59). While LDPI can provide a perfusion index of the ischemic tissues relative to control limbs, the time required for the scan and the artifactual movement of red blood cells would preclude its use during muscle contraction, and thus in assessing peak functional hyperemia. In addition, LDPI provides only a measurement of the most superficial tissues and may not represent whole limb and especially deep muscles.

Microspheres have been considered the gold standard for measurement of blood flow and can provide measurement of flow in all individual muscles. They have been successfully used in rat hindlimb models of arterial insufficiency, represent an outstanding method to assess muscle perfusion(16), and can be utilized in unanesthetized animals during treadmill running(60). However, the microsphere technique also has limitations(16), including: processing time, potential loss and variability, and difficulties with the technique related to consistency and interpretation of results when applied to a rat hindlimb model of ischemia(61). It also is invasive, technically challenging(55), and does not permit real time or longitudinal studies. In addition, collection of the reference blood samples necessary to provide absolute perfusion values may not be possible in small rodents.

In this study, we elected to evaluate the venous outflow method in combination with local skeletal muscle stimulation as an alternative technique to assess flow capacity or functional reserve. Our results with the venous outflow technique demonstrate it to be highly reproducible and sufficient to detect differences in flow capacity during various levels of stimulation between healthy rodents and those with vascular risk factors, and also capable of assessing the efficacy of different therapies. Placement of the flow probe on the femoral vein draining the calf muscles (Figure 1) minimizes the contribution of flow from muscles

outside the collateral-dependent region. This was a major criticism of previous studies which measured flow in major inflow arteries proximal to the site of arterial ligation(15). While some of the measured venous flow may originate from the distal thigh muscles outside the collateral dependent region, the contribution during exercise is minimized by selective contraction of the calf muscles. Furthermore, any contribution from the thigh muscles would lead to an overestimate of calf perfusion which should also be present in the control limb. Our studies demonstrate that lower limb maximal perfusion remains suppressed and that the venous outflow technique is capable of unmasking perfusion deficits consistent with what has been reported with microspheres in calf muscles(16). However, the method is invasive and requires surgical training and experience to prevent complications including vascular injury. We did not experience complications and others have reported successful acute and chronic utilization of the miniature flow probes on the mouse artery(37–40). Our preliminary studies in the mouse (Figure 5) suggest that the venous outflow technique could be utilized in this species as well.

4.5 Conclusion and Future application

In conclusion, we have presented the measurement of venous outflow with transit time ultrasound flow probes during electrically stimulated contraction of calf muscles as a novel method to assess flow capacity in the distal limb of small rodents with subcritical limb ischemia. The method is demonstrated to be highly reproducible and sufficient to unmask perfusion deficits that are not apparent at rest and can be utilized to assess responses to different stimulus levels that might represent mild to intense skeletal muscle activity. It is capable of detecting impaired compensation in animal models of arterial disease and assessing therapeutic efficacy, including when such therapy is not initiated until after a stable response to the occlusive insult is achieved. The novel approach provides an alternative to the existing techniques including LDPI which provides longitudinal studies but cannot assess functional flow capacity and microspheres which some investigators have found difficult to adapt to small rodents. The venous outflow method combined with stimulation of calf muscle contraction provides a new tool for the assessment of subcritical limb ischemia and may facilitate preclinical assessment of novel therapies in small rodents. We suggest that future applications of this technique should be combined with histological studies to assess muscular and microvascular adaptations, muscle function studies, and proximal and distal pressure measurements to assess hemodynamic significance of collateral vs. distal adaptations. Future studies should also include sham operated controls to establish the extent to which surgical manipulations during model creation influence results.

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References

1. Criqui MH, Langer RD, Fronek A, Feigelson HS, Klauber MR, McCann TJ, Browner D. Mortality over a Period of 10 Years in Patients with Peripheral Arterial Disease. N Engl J Med. 1992; 326:381–386. [PubMed: 1729621]

2. Golomb BA, Dang TT, Criqui MH. Peripheral Arterial Disease: Morbidity and Mortality Implications. Circulation. 2006; 114:688–699. [PubMed: 16908785]
3. Allison MA, Ho E, Denenberg JO, Langer RD, Newman AB, Fabsitz RR, Criqui MH. Ethnic-Specific Prevalence of Peripheral Arterial Disease in the United States. Am J Prev Med. 2007; 32:328–333. [PubMed: 17383564]
4. Hankey GJ, Norman PE, Eikelboom JW. Medical treatment of peripheral arterial disease. JAMA. 2006; 295:547–553. [PubMed: 16449620]
5. Criqui MH, Fronek A, Barrett-Connor E, Klauber MR, Gabriel S, Goodman D. The prevalence of peripheral arterial disease in a defined population. Circulation. 1985; 71:510–515. [PubMed: 3156006]
6. Meijer WT, Grobbee DE, Hunink M, Hofman A, Hoes AW. Determinants of peripheral arterial disease in the elderly: The rotterdam study. Arch Intern Med. 2000; 160:2934–2938. [PubMed: 11041900]
7. Hirsch AT, Duval S. The global pandemic of peripheral artery disease. The Lancet. 382:1312–1314.
8. Fowkes FGR, Rudan D, Rudan I, Aboyans V, Denenberg JO, McDermott MM, Norman PE, Sampson UKA, Williams LJ, Mensah GA, Criqui MH. Comparison of global estimates of prevalence and risk factors for peripheral artery disease in 2000 and 2010: a systematic review and analysis. The Lancet. 2013; 382:1329–1340.
9. Hirsch AT, Criqui MH, Treat-Jacobson D, et al. Peripheral arterial disease detection, awareness, and treatment in primary care. JAMA. 2001; 286:1317–1324. [PubMed: 11560536]
10. Muluk SC, Muluk VS, Kelley ME, Whittle JC, Tierney JA, Webster MW, Makaroun MS. Outcome events in patients with claudication: A 15-year study in 2777 patients. J Vasc Surg. 2001; 33:251–258. [PubMed: 11174775]
11. Gornik HL. Rethinking the morbidity of peripheral arterial disease and the “normal” ankle-brachial index. J Am Coll Cardiol. 2009; 53:1063–1064. [PubMed: 19298920]
12. Ahimastos AA, Walker PJ, Askew C, et al. Effect of ramipril on walking times and quality of life among patients with peripheral artery disease and intermittent claudication: A randomized controlled trial. JAMA. 2013; 309:453–460. [PubMed: 23385271]
13. McDermott M. Medications for improving walking performance in peripheral artery disease: Still miles to go. JAMA. 2013; 309:487–488. [PubMed: 23385276]
14. Ahimastos AA, Lawler A, Reid CM, Blombery PA, Kingwell BA. Brief Communication: Ramipril Markedly Improves Walking Ability in Patients with Peripheral Arterial Disease: A Randomized Trial. Ann Intern Med. 2006; 144:660–664. [PubMed: 16670135]
15. Waters RE, Terjung RL, Peters KG, Annex BH. Preclinical models of human peripheral arterial occlusive disease: implications for investigation of therapeutic agents. J Appl Physiol. 2004; 97:773–780. [PubMed: 15107408]
16. Brevetti LS, Paek R, Brady SE, Hoffman JI, Sarkar R, Messina LM. Exercise-induced hyperemia unmasks regional blood flow deficit in experimental hindlimb ischemia. J Surg Res. 2001; 98:21–26. [PubMed: 11368533]
17. Corcoran HA, Smith BE, Mathers P, Pisacreta D, Hershey JC. Laser Doppler imaging of reactive hyperemia exposes blood flow deficits in a rat model of experimental limb ischemia. J Cardiovasc Pharmacol. 2009; 53:446–451. [PubMed: 19433986]
18. You D, Cochain C, Loinard C, Vilar J, Mees B, Duriez M, Levy BI, Silvestre JS. Hypertension Impairs Postnatal Vasculogenesis: Role of Antihypertensive Agents. Hypertension. 2008; 51:1537–1544. [PubMed: 18426993]
19. Iaccarino G, Ciccarelli M, Sorriento D, Galasso G, Campanile A, Santulli G, Cipolletta E, Cerullo V, Cimini V, Altobelli GG, Piscione F, Priante O, Pastore L, Chiariello M, Salvatore F, Koch WJ, Trimarco B. Ischemic Neoangiogenesis Enhanced by {beta}2-Adrenergic Receptor Overexpression: A Novel Role for the Endothelial Adrenergic System. Circ Res. 2005; 97:1182–1189. [PubMed: 16239589]
20. Srivastava S, Terjung RL, Yang HT. Basic fibroblast growth factor increases collateral blood flow in spontaneously hypertensive rats. Am J Physiol Heart Circ Physiol. 2003; 285:H1190–H1197. [PubMed: 12763749]

21. Emanueli C, Salis MB, Stacca T, Pinna A, Gaspa L, Spano A, Madeddu P. Ramipril improves hemodynamic recovery but not microvascular response to ischemia in spontaneously hypertensive rats. Am J Hypertens. 2002; 15:410–415. [PubMed: 12022243]
22. Emanueli C, Salis MB, Stacca T, Gaspa L, Chao J, Chao L, Piana A, Madeddu P. Rescue of Impaired Angiogenesis in Spontaneously Hypertensive Rats by Intramuscular Human Tissue Kallikrein Gene Transfer. Hypertension. 2001; 38:136–141. [PubMed: 11463774]
23. Troidl K, Tribulova S, Cai WJ, Ruding I, Apfelbeck H, Schierling W, Troidl C, Schmitz-Rixen T, Schaper W. Effects of endogenous nitric oxide and of DETA NONOate in arteriogenesis. J Cardiovasc Pharmacol. 2010; 55:153–160. [PubMed: 20173509]
24. Matsumura M, Fukuda N, Kobayashi N, Umezawa H, Takasaka A, Matsumoto T, Yao EH, Ueno T, Negishi N. Effects of atorvastatin on angiogenesis in hindlimb ischemia and endothelial progenitor cell formation in rats. J Atheroscler Thromb. 2009; 16:319–326. [PubMed: 19672036]
25. Scheidegger KJ, Nelissen-Vrancken MH, Leenders PJ, Daemen MJ, Smits JF, Wood JM. Structural adaptation to ischemia in skeletal muscle: effects of blockers of the renin-angiotensin system. J Hypertens. 1997; 15:1455–1462. [PubMed: 9431852]
26. Nelissen-Vrancken HJ, Boudier HA, Daemen MJ, Smits JF. Antihypertensive therapy and adaptive mechanisms in peripheral ischemia. Hypertension. 1993; 22:780–788. [PubMed: 8225538]
27. Nelissen-Vrancken HJ, Leenders PJ, Struijker Boudier HA, Smits JF. Increased responsiveness of the vascular bed to angiotensin I, angiotensin II and phenylephrine in acute and chronic ischemic hindlimbs in rats. J Vasc Res. 1992; 29:359–366. [PubMed: 1330017]
28. Miller SJ, Norton LE, Murphy MP, Dalsing MC, Unthank JL. The Role of the Renin-Angiotensin System and Oxidative Stress in Spontaneously Hypertensive Rat (SHR) Mesenteric Collateral Growth Impairment. Am J Physiol Heart Circ Physiol. 2007; 292:H2523–H2531. [PubMed: 17277018]
29. Tuttle JL, Sanders BM, Burkhart HM, Fath SW, Herring BP, Dalsing MC, Unthank JL. Impaired collateral artery development in spontaneously hypertensive rats. Microcirculation. 2002; 9:343–351. [PubMed: 12375172]
30. Li TS, Furutani A, Takahashi M, Ohshima M, Qin SL, Kobayashi T, Ito H, Hamano K. Impaired potency of bone marrow mononuclear cells for inducing therapeutic angiogenesis in obese diabetic rats. AJP - Heart and Circulatory Physiology. 2006; 290:H1362–H1369. [PubMed: 16227342]
31. Janiak P, Lainee P, Grataloup Y, Luyt CE, Bidouard JP, Michel JB, O'Connor SE, Herbert JM. Serotonin receptor blockade improves distal perfusion after lower limb ischemia in the fatty Zucker rat. Cardiovasc Res. 2002; 56:293–302. [PubMed: 12393100]
32. Yang HT, Terjung RL. Angiotensin-converting enzyme inhibition increases collateral-dependent muscle blood flow. J Appl Physiol. 1993; 75:452–457. [PubMed: 8397181]
33. Distasi MR, Case J, Ziegler MA, Dinauer MC, Yoder MC, Haneline LS, Dalsing MC, Miller SJ, Labarrere CA, Murphy MP, Ingram DA, Unthank JL. Suppressed hindlimb perfusion in Rac2−/− and Nox2−/− mice does not result from impaired collateral growth. Am J Physiol Heart Circ Physiol. 2009; 296:H877–886. [PubMed: 19151256]
34. Coffman JD. Peripheral collateral blood flow and vascular reactivity in the dog. The Journal of Clinical Investigation. 1966; 45:923–931. [PubMed: 5913300]
35. Sanne H, Sivertsson R. The effect of exercise on the development of collateral circulation after experimental occlusion of the femoral artery in the cat. Acta Physiol Scand. 1968; 73:257–263. [PubMed: 5709583]
36. Welch WJ, Deng X, Snellen H, Wilcox CS. Validation of miniature ultrasonic transit-time flow probes for measurement of renal blood flow in rats. Am J Physiol. 1995; 268:F175–178. [PubMed: 7840243]
37. Cabou C, Cani PD, Campistron G, Knauf C, Mathieu C, Sartori C, Amar J, Scherrer U, Burcelin R. Central Insulin Regulates Heart Rate and Arterial Blood Flow: An Endothelial Nitric Oxide Synthase–Dependent Mechanism Altered During Diabetes. Diabetes. 2007; 56:2872–2877. [PubMed: 17804761]
38. Cabou C, Campistron G, Marsollier N, Leloup C, Cruciani-Guglielmacci C, Penicaud L, Drucker DJ, Magnan C, Burcelin R. Brain Glucagon-Like Peptide-1 Regulates Arterial Blood Flow, Heart Rate, and Insulin Sensitivity. Diabetes. 2008; 57:2577–2587. [PubMed: 18633100]

39. Sonobe T, Tsuchimochi H, Schwenke DO, Pearson JT, Shirai M. Treadmill running improves hindlimb arteriolar endothelial function in type 1 diabetic mice as visualized by X-ray microangiography. Cardiovasc Diabetol. 2015; 14:51. [PubMed: 25964060]
40. Wang CH, Chen KT, Mei HF, Lee JF, Cherng WJ, Lin SJ. Assessment of mouse hind limb endothelial function by measuring femoral artery blood flow responses. J Vasc Surg. 2011; 53:1350–1358. [PubMed: 21276693]
41. D'Almeida MS, Gaudin C, Lebrec D. Validation of 1- and 2-mm transit-time ultrasound flow probes on mesenteric artery and aorta of rats. American Journal of Physiology - Heart and Circulatory Physiology. 1995; 268:H1368–H1372.
42. Lundell A, Bergqvist D, Mattsson E, Nilsson B. Volume blood flow measurements with a transit time flowmeter: an in vivo and in vitro variability and validation study. Clin Physiol. 1993; 13:547–557. [PubMed: 8222539]
43. D'Almeida MS, Cailmail S, Lebrec D. Validation of transit-time ultrasound flow probes to directly measure portal blood flow in conscious rats. American Journal of Physiology - Heart and Circulatory Physiology. 1996; 271:H2701–H2709.
44. Beldi G, Bosshard A, Hess OM, Althaus U, Walpoth BH. Transit time flow measurement: experimental validation and comparison of three different systems. The Annals of Thoracic Surgery. 2000; 70:212–217. [PubMed: 10921710]
45. Lash JM. Arterial and arteriolar contributions to skeletal muscle functional hyperemia in spontaneously hypertensive rats. J Appl Physiol. 1995; 78:93–100. [PubMed: 7713849]
46. Tyml K. Red cell perfusion in skeletal muscle at rest and after mild and severe contractions. American Journal of Physiology - Heart and Circulatory Physiology. 1987; 252:H485–H493.
47. Honig CR, Odoroff CL, Frierson JL. Capillary recruitment in exercise: rate, extent, uniformity, and relation to blood flow. American Journal of Physiology - Heart and Circulatory Physiology. 1980; 238:H31–H42.
48. Walder CE, Errett CJ, Bunting S, Lindquist P, Ogez JR, Heinsohn HG, Ferrara N, Thomas GR. Vascular endothelial growth factor augments muscle blood flow and function in a rabbit model of chronic hindlimb ischemia. J Cardiovasc Pharmacol. 1996; 27:91–98. [PubMed: 8656665]
49. Ziegler MA, Distasi MR, Bills RG, Miller SJ, Alloosh M, Murphy MP, George Akingba A, Sturek M, Dalsing MC, Unthank JL. Marvels, mysteries, and misconceptions of vascular compensation to peripheral artery occlusion. Microcirculation. 2010; 17:3–20. [PubMed: 20141596]
50. Capoccia BJ, Shepherd RM, Link DC. G-CSF and AMD3100 mobilize monocytes into the blood that stimulate angiogenesis in vivo through a paracrine mechanism. Blood. 2006; 108:2438–2445. [PubMed: 16735597]
51. Zbinden S, Clavijo LC, Kantor B, Morsli H, Cortes GA, Andrews JA, Jang GJ, Burnett MS, Epstein SE. Interanimal variability in preexisting collaterals is a major factor determining outcome in experimental angiogenesis trials. AJP - Heart and Circulatory Physiology. 2007; 292:H1891–H1897. [PubMed: 17189353]
52. Emanueli C, Salis MB, Stacca T, Pinna A, Gaspa L, Madeddu P. Angiotensin AT(1) receptor signalling modulates reparative angiogenesis induced by limb ischaemia. Br J Pharmacol. 2002; 135:87–92. [PubMed: 11786483]
53. Levy BI. How to Explain the Differences Between Renin Angiotensin System Modulators. Am J Hypertens. 2005; 18:134–141.
54. Munzel T, Keaney JF. Are ACE Inhibitors a "Magic Bullet" Against Oxidative Stress? Circulation. 2001; 104:1571–1574. [PubMed: 11571254]
55. Lotfi S, Patel AS, Mattock K, Egginton S, Smith A, Modarai B. Towards a more relevant hind limb model of muscle ischaemia. Atherosclerosis. 2013; 227:1–8. [PubMed: 23177969]
56. Hoefer IE, van Royen N, Rectenwald JE, Bray EJ, Abouhamze Z, Moldawer LL, Voskuil M, Piek JJ, Buschmann IR, Ozaki CK. Direct Evidence for Tumor Necrosis Factor-α Signaling in Arteriogenesis. Circulation. 2002; 105:1639–1641. [PubMed: 11940540]
57. Radegran G. Limb and skeletal muscle blood flow measurements at rest and during exercise in human subjects. Proc Nutr Soc. 1999; 58:887–898. [PubMed: 10817156]

58. Chiba M, Nakamura M, Kanaya Y, Kobayashi N, Ueshima K, Kawazoe K, Hiramori K. Improvement in lower limb vasodilatory reserve and exercise capacity in patients with chronic heart failure due to valvular heart disease. Eur Heart J. 1997; 18:1931–1936. [PubMed: 9447321]

59. Schmidt MA, Chakrabarti A, Shamim-Uzzaman Q, Kaciroti N, Koeppe RA, Rajagopalan S. Calf Flow Reserve with H215O PET as a Quantifiable Index of Lower Extremity Flow. J Nucl Med. 2003; 44:915–919. [PubMed: 12791819]

60. Yang HT, Ren J, Laughlin MH, Terjung RL. Prior exercise training produces NO-dependent increases in collateral blood flow after acute arterial occlusion. Am J Physiol Heart Circ Physiol. 2002; 282:H301–H310. [PubMed: 11748075]

61. Lundberg G, Luo F, Blegen H, Kalin B, Wahlberg E. A rat model for severe limb ischemia at rest. Eur Surg Res. 2003; 35:430–438. [PubMed: 12928601]

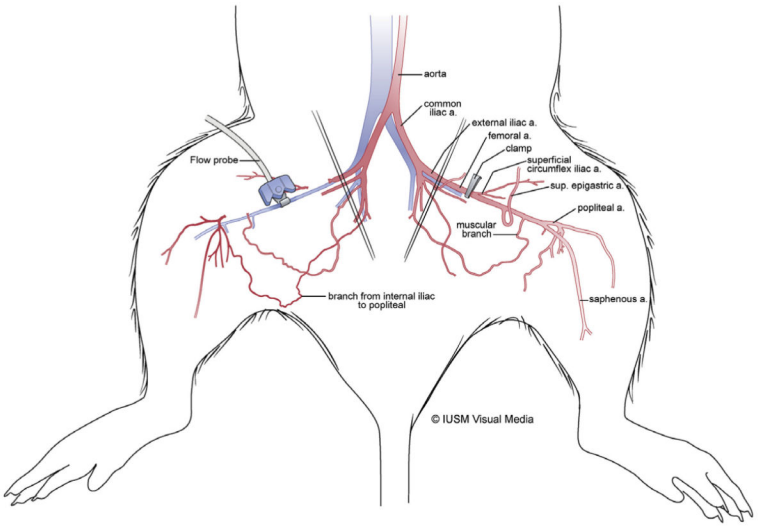


Figure 1.

Line drawing representing hindlimb models used in this investigation. Major branches of the iliac-femoral arterial system of the rat from its origin at the aortic trifurcation (tail artery not shown) to its division into the saphenous and popliteal arteries of the distal limb are represented. In all experiments, the left limb served as the control. This control limb was acutely occluded with a microvascular clamp approximately 5 mm distal to the inguinal ligament to determine the degree to which blood flow was compromised at rest and during skeletal muscle contraction. The femoral artery of the right limb was either ligated (WKY) at the same location where the clamp was applied to the control limb or ligated and excised in lean and obese Zucker and SHR rats to be consistent with previous studies.

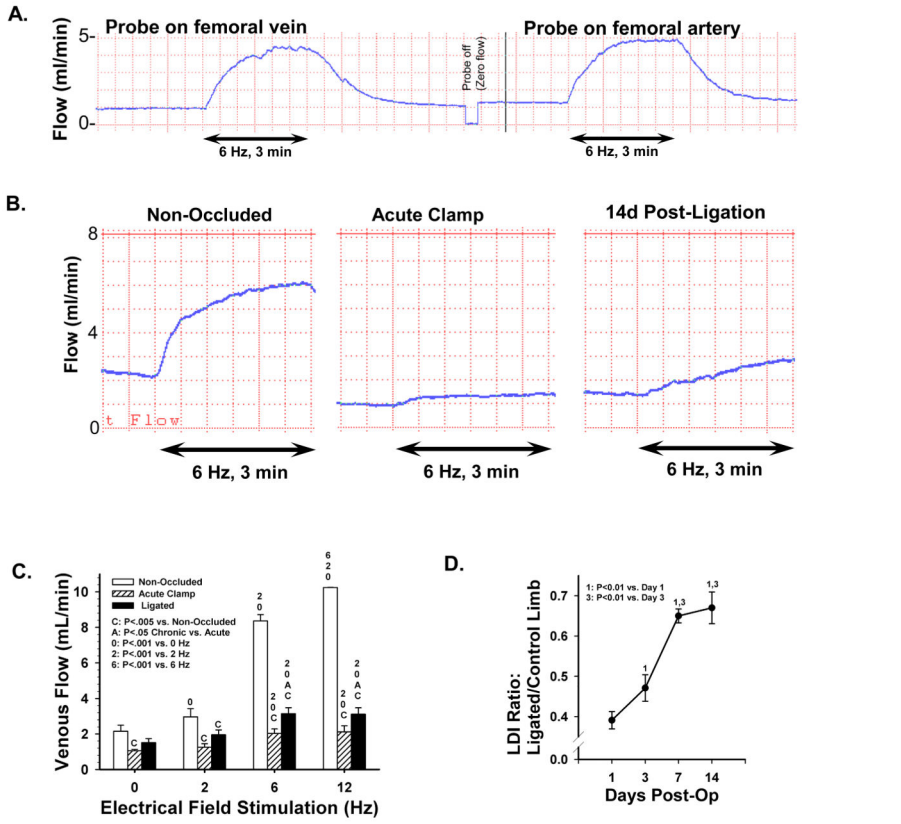
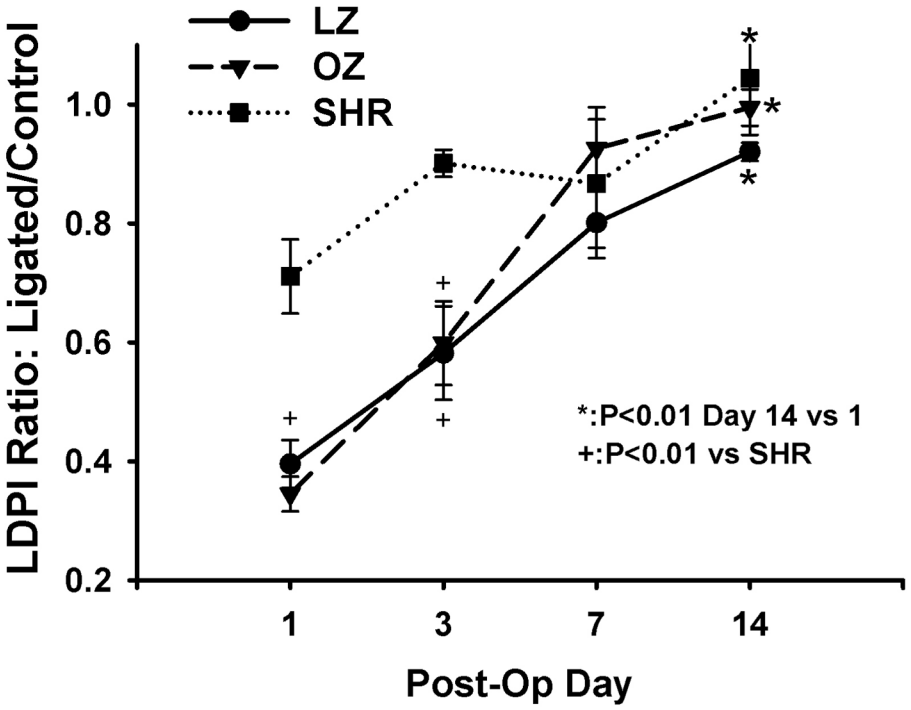
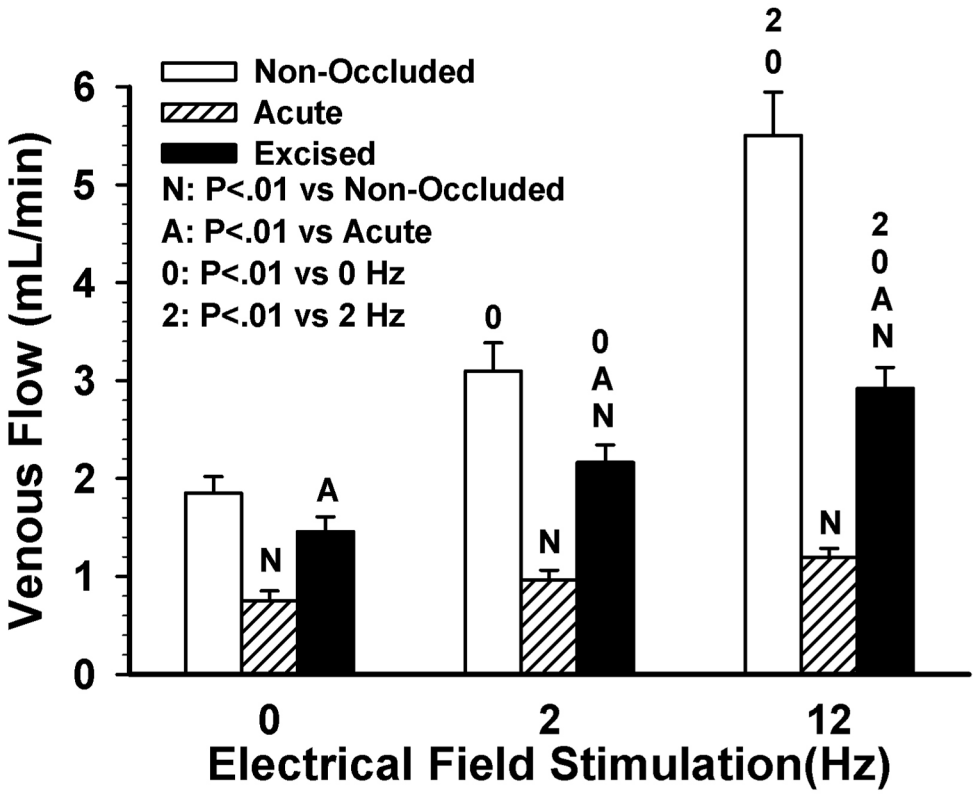


Figure 2.

Development and validation of combined venous outflow and skeletal muscle stimulation to assess blood flow reserve. A. Typical tracings of femoral vein and artery blood flow from rest to contraction-induced hyperemia and recovery in WKY. B. Representative records of venous outflow before and during skeletal muscle contraction induced by electrical field stimulation in the left limb before (Non-Occluded) and after acute (Acute) femoral artery occlusion with microvascular clamp (left and center panels, respectively) and in the right limb with femoral artery ligation 2 weeks previous (Ligated) (right panel). C. Average venous outflow at rest and during graded contraction induced by electrical field stimulation. D. Average LDPI of experimental limb over time relative to same animal control limb.





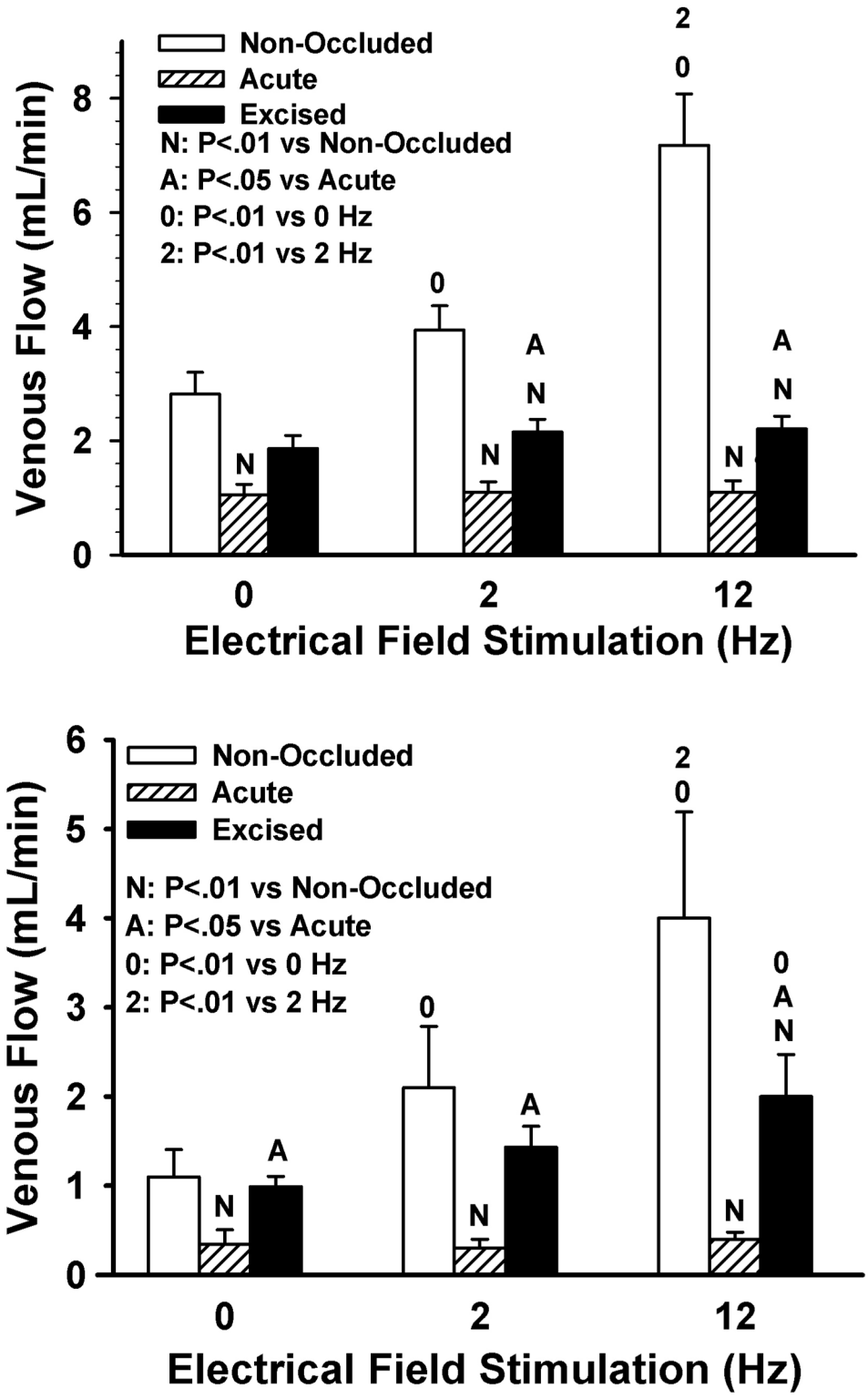


Figure 3.

Comparison of laser Doppler perfusion imaging (LDPI, panel A) and venous outflow at rest and during stimulated muscle contraction in control, lean Zucker rats (LZR, panel B), obese

Zucker rats (OZR, panel C), and Spontaneously Hypertensive rats (SHR, panel D). Venous flow measurements were made 2 weeks following femoral artery excision.

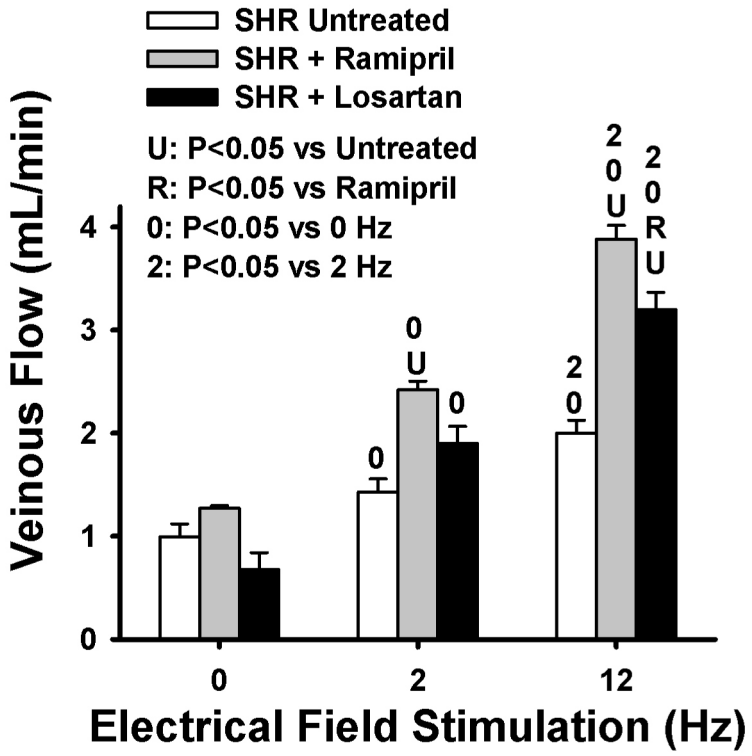


Figure 4.

Effect of pharmacological therapy (Ramipril and Losartan) on blood flow reserve in the limb of SHR. Therapy was initiated 2 weeks after femoral artery excision and continued for two weeks at which time the flow measurements were performed.

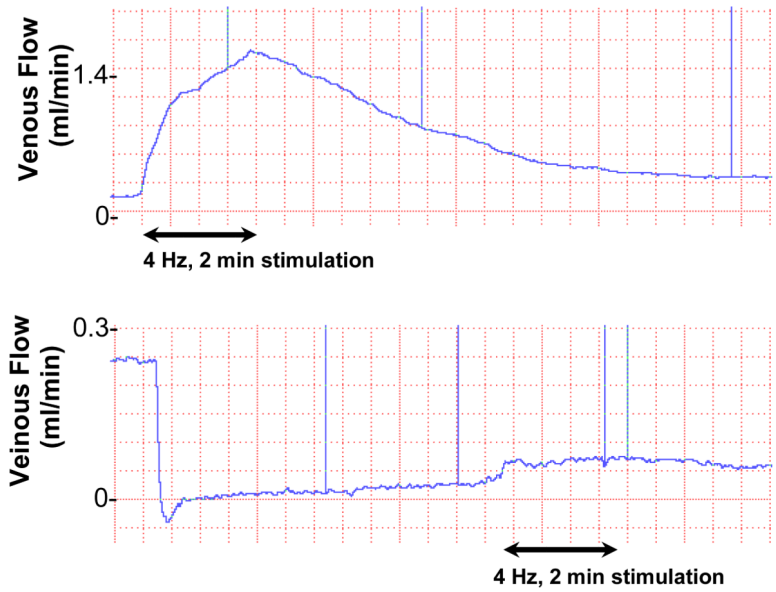


Figure 5.

Application of the venous outflow and skeletal muscle stimulation technique in mice. A) Illustrates the increase in flow from 0.10 ml/min at rest to a peak of about 1.13 ml/min during 4 Hz stimulation for 2 min. The tracing in B) is a magnified continuation of that in A) and shows the initial drop in blood flow with acute ligation of the distal femoral artery, a slight recovery that would be expected with dilation of the vasculature, and a greatly suppressed hyperemic response with stimulation. Similar results were observed in acute experiments on 4 mice. In conclusion, the venous outflow protocol presented here is feasible in mice but is technically challenging and requires more work to fully quantify and validate the technique.

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Table 1

Average body mass and mean arterial pressure (MAP)

	Size (n)	Body Mass (g)	MAP (mm Hg)
WKY	5	258±4.4	140±6.7
LZR	8	322±7.7	149±2.9
OZR	7	531±12.0	161±4.2
SHR	7	404±14.0	197±1.9
SHR + Ramipril	3	418±15.1	135±6.9
SHR + Losartan	4	398±20.2	161±4.7